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Small heat shock protein AgsA forms dynamic fibrils

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ABSTRACT

As a class of molecular chaperones, small heat shock proteins (sHsps) usually exist as multi-subunit spherical oligomers. In this study, we report that AgsA, a sHsp of *Salmonella enterica* serovar Typhimurium, spontaneously forms fibrils *in vitro*. These fibrils tend to be formed at elevated temperature and do not share the characteristics of amyloid. Interestingly, the fibril-forming AgsA is able to suppress the dithiothreitol-induced aggregation of insulin efficiently within a certain range of temperature. During this process, AgsA fibrils disappear and spherical complexes form between AgsA and insulin molecules. These data suggest that AgsA fibrils may represent a distinctive type of structural and functional form of sHsp from spherical oligomers. Our study provides new insights into sHsp structures and chaperone functions.

Structured summary of protein interactions:
AgsA and AgsA **bind** by **electron microscopy (View interaction).**
Insulin and Insulin **bind** by **molecular sieving (View interaction).**
Insulin and Insulin **bind** by **electron microscopy (View interaction).**
AgsA and AgsA **bind** by **molecular sieving (View interaction).**
AgsA and AgsA **bind** by **fluorescence technology (View interaction).**
AgsA and AgsA **bind** by **circular dichroism (View interaction).**
Insulin and Insulin **bind** by **fluorescence technology (View interaction).**
AgsA and Insulin **bind** by **molecular sieving (View interaction).**
AgsA and AgsA **bind** by **electron tomography (View interaction).**

1. Introduction

Small heat shock proteins (sHsps) are ubiquitous stress proteins of 12–43 kDa that are characterized by a conserved α-crystallin domain containing approximately 100 amino acid residues [1,2]. In general, sHsps are present as multi-subunit spherical oligomers *in vitro* [1,2]. As a class of molecular chaperone, sHsp prevents irreversible protein aggregation under stress conditions in an ATP-independent manner by holding the partially folded intermediates, which can be subsequently refolded by ATP-dependent chaperones, such as Hsp60, Hsp70 and Hsp100 [3–7]. The action mechanism for the chaperone activity of sHsps is postulated to be the stress-induced exposure of hydrophobic patches, which are likely to serve as the binding sites for the partially misfolded client proteins [8–10]. Mostly, sHsps undergo a significant increase in chaperone-like activity at heat shock temperature, suspected resulting from the reversible dissociation of the large oligomers of sHsps [11,12].

Bacteria, such as *Escherichia coli*, generally possess one or two conserved sHsps [13]. However, *Salmonella enterica* serovar Typhimurium encodes a third one, i.e. AgsA (aggregation-suppressing protein), which is strongly induced at high temperature [14]. Overproduction of AgsA partially complements the thermosensitive phenotype of *E. coli dnaK* null mutant and prevents the aggregation of denatured proteins in *dnaK* and *rpoH* null mutant cells [14]. Therefore, AgsA is believed to function as a cytosolic chaperone. *In vitro* studies have demonstrated that AgsA adopts large oligomeric structure and possesses strong chaperone-like activity at high temperature [15].

Unlike a number of sHsp oligomers, AgsA oligomers fail to dissociate at temperature as high as 50 °C. Rather, a decrease

Abbreviations: sHsp, small heat shock protein; AgsA, aggregation-suppressing protein; DTT, dithiothreitol; SEC, size exclusion chromatography; ThT, thioflavin T; EM, electron microscopy; CR, Congo Red; CD, circular dichroism

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of such oligomers was observed upon a temperature increase from 25 to 50 °C, as revealed by size exclusion chromatography (SEC) [15]. This unusual observation raises the questions as to whether AgsA aggregates at elevated temperature, and if not, what structural forms it really adopts. Here we provide evidence, mainly by utilizing electron microscopy (EM), to show that AgsA neither undergoes oligomeric dissociation nor aggregation, but forms fibrils. The fibrils formed by this protein do not share characteristics of amyloids. Importantly, data in this study suggest that fibrils may represent a distinctive type of structural and functional form of AgsA from spherical oligomers, thus providing new insights into sHsp structures and chaperone functions.

2. Materials and methods

2.1. Plasmid construction and protein purification

The DNA sequence encoding AgsA was synthesized by Aoke Company in Beijing, and then sub-cloned into pET-21a. The recombinant plasmid was transformed into *E. coli* strain BL21 (DE3) cells for protein expression under the induction of 1 mM isopropyl β-D-1-thiogalactopyranoside for 6 h at 37 °C. Cell pellets were obtained after centrifugation at 5000×*g* for 10 min at 4 °C and re-suspended in buffer A (20 mM Tris–HCl, pH 8.0) containing 1 mM protease inhibitor PMSF (Boehringer Mannheim). The cells were lysed by ultra-sonication and then centrifuged at 15000×g for 40 min at 4 °C to obtain the supernatant that was immediately loaded onto HiTrap Q Sepharose column pre-equilibrated with buffer A. After being washed with approximately one column volume of buffer A, the protein was eluted using a salt gradient of 0–1.0 M NaCl. Fractions containing AgsA, identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), were pooled and dialyzed against buffer B (50 mM sodium phosphate, pH 7.5). After an addition of 1.5 M $(NH_4)_2SO_4$, the samples were centrifuged at 15000×*g* for 40 min at 4 °C. The supernatant was loaded onto phenyl-Sepharose HP and eluted with a salt gradient of 1.5–0 M $(NH_4)_2SO_4$. The fractions containing AgsA protein, confirmed by SDS–PAGE analysis, were subjected to desalting via HiPrep 26/10 column. Protein concentration was measured by the BCA assay (Pierce) according to the manufacturer's instructions.

2.2. Electron microscopy

To observe the AgsA fibrils, AgsA (0.3 mg/ml) was incubated in buffer (20 mM Tris–HCl, pH 8.0) at indicated temperatures for 30 min and then applied to carbon-coated copper grids. The binding of AgsA to the grids were conducted by placing the grids on a parafilm floating on the water surface in a closed water-bath heater preset to the testing temperature. After incubation for 1 min, excess solution was removed with filter paper. Grids were negatively stained with 1% (w/v) uranyl acetate for 30 s. Electron micrographs were recorded using a JEOL 1230 transmission electron microscope. To observe the AgsA–insulin complex, AgsA (0.3 mg/ml) was incubated in buffer (20 mM Tris–HCl, pH 8.0) at indicated temperatures for 30 min to form fibrils and then incubated with insulin (0.3 mg/ml) in the presence of dithiothreitol (DTT) (20 mM) at the same temperatures for another 30 min. The samples were centrifuged at 15000×*g* for 1 min at 4 °C, and then applied to carbon-coated copper grids. After incubation for 1 min, excess solution was removed with filter paper. Grids were negatively stained with 1% (w/v) uranyl acetate for 30 s. Electron micrographs were recorded using a JEOL 1230 transmission electron microscope.

2.3. Cryo-electron tomography of fibril structures of AgsA and measurement of fiber length and diameter

In order to accurately determine the length and the diameter of fibrils, cryo-electron tomography was performed to reconstruct the 3D volume of fibrils. Protein solution (3 mg/ml) was incubated at different temperatures for 30 min, and then deposited onto freshly glow-discharged holey carbon grids for 20 s after mixing with 6 nm gold clusters. The grids were blotted and rapidly frozen in liquid ethane maintained at −180 °C using FEI Virobot IV. The resultant frozen-hydrated specimens were imaged at −170 °C using a JEOL2100 or JEOL2200F electron microscope. The data were collected at a magnification of 30000×, resulting in an effective pixel size of 2.8 Å. Using the Serial EM program, we collected low-dose single-axis tilt series from each temperatures at a defocus of −4 to −6 μm with a cumulative dose of ~100 $e^-/Å^2$ distributed over 60 images, covering an angular range from −60° to +60° at angular increments of 1.5°–2°. Tilted images were aligned using fiducial markers and 3D volumes were reconstructed using the IMOD software package. In total, eight cryotomograms from each temperature were collected and reconstructed. Results of reconstruction were then low-pass filtered by EMAN2 software package. Fibril structures in each tomogram were labeled and measured in Amira.

2.4. Thioflavin T fluorescence assay

Thioflavin T (ThT) fluorescence assay was conducted according to Meehan et al. [16]. ThT (a final dye concentration of 50 μM) was incubated with AgsA protein solution (0.1 mg/ml) in 10 mM phosphate, 150 mM NaCl, adjusted to pH 7.0. Fresh solutions of ThT were passed through a 0.2 μm filter immediately before use. A Hitachi F4500 fluorescence spectrophotometer was used to record the spectra with 1 cm path length cuvettes (λ_{Ex} = 442 nm; λ_{Em} = 460–550 nm). An increase in the fluorescence emission intensity at 490 nm was taken to be indicative of amyloid formation.

2.5. Congo Red (CR) assay

Congo Red fluorescence assay was conducted according to Meehan et al. [16]. Fresh solutions of Congo Red (CR) in 5 mM potassium phosphate buffer containing 150 mM NaCl, pH 7.4, were passed through a 0.2 μm filter immediately before use. The CR solutions were added to 100 μg/ml protein solutions to a final dye concentration of 5 μM, and the samples vortexed for 15 s. The absorption spectrum of each sample was recorded from 400 to 700 nm on a UV-8500 spectrophotometer (Shanghai TechCorp). The spectrum of CR alone was compared with that of CR solutions in the presence of protein. A red shift of the absorption band toward 540 nm and an increase in absorption were together taken to be indicative of the formation of amyloid structures.

2.6. Insulin and lysozyme amyloid formation

Insulin amyloid was grown according to Wang et al. [17]. Briefly, bovine insulin was dissolved in acidic medium (100 mM NaCl, pH 1.5, adjusted by HCl) at a final concentration of 1 mg/ml. To form amyloid, the insulin solution was incubated at 50 °C for 20 h before ThT fluorescence assay. Lysozyme amyloid was grown according to Hu et al. [18]. Briefly, lysozyme was dissolved in acidic medium (100 mM NaCl, pH 2.0, adjusted by HCl) at a final concentration of 10 mg/ml. To form amyloid, the lysozyme solution was incubated at 37 °C for a week before CR assay.

2.7. Far-UV circular dichroism (CD) assay

Far-UV CD assay was performed in a J-715 spectropolarimeter (JASCO, Japan) equipped with a constant-temperature water bath.

A sample of 300 μl AgsA (0.2 mg/ml, 12.5 mM sodium phosphate buffer, pH 7.5) was added into a 2-mm quartz cuvette and scanned after being equilibrated at indicated temperatures for 30 min to form fibrils. Each spectrum represents an average of three such runs.

2.8. In vitro chaperone-like activity assay of AgsA

The chaperone-like activity was assayed by measuring the capacity of AgsA to suppress the DTT-induced aggregation of insulin at increasing temperatures, which was monitored on a UV-8500 spectrophotometer (Shanghai TechCorp) at 360 nm. The final concentrations of DTT and insulin were 20 mM and 0.5 mg/ml respectively. Before chaperone-like activity assay, samples of AgsA were incubated for 30 min at testing temperatures to form fibrils. The chaperone-like activity was defined by the percentage of the aggregation of DTT-treated insulin that was inhibited by the presence of AgsA (0.5 mg/ml) at testing temperatures $((A360^{\text{insulin alone}} - A360^{\text{insulin + AgsA}})/A360^{\text{insulin alone}})$.

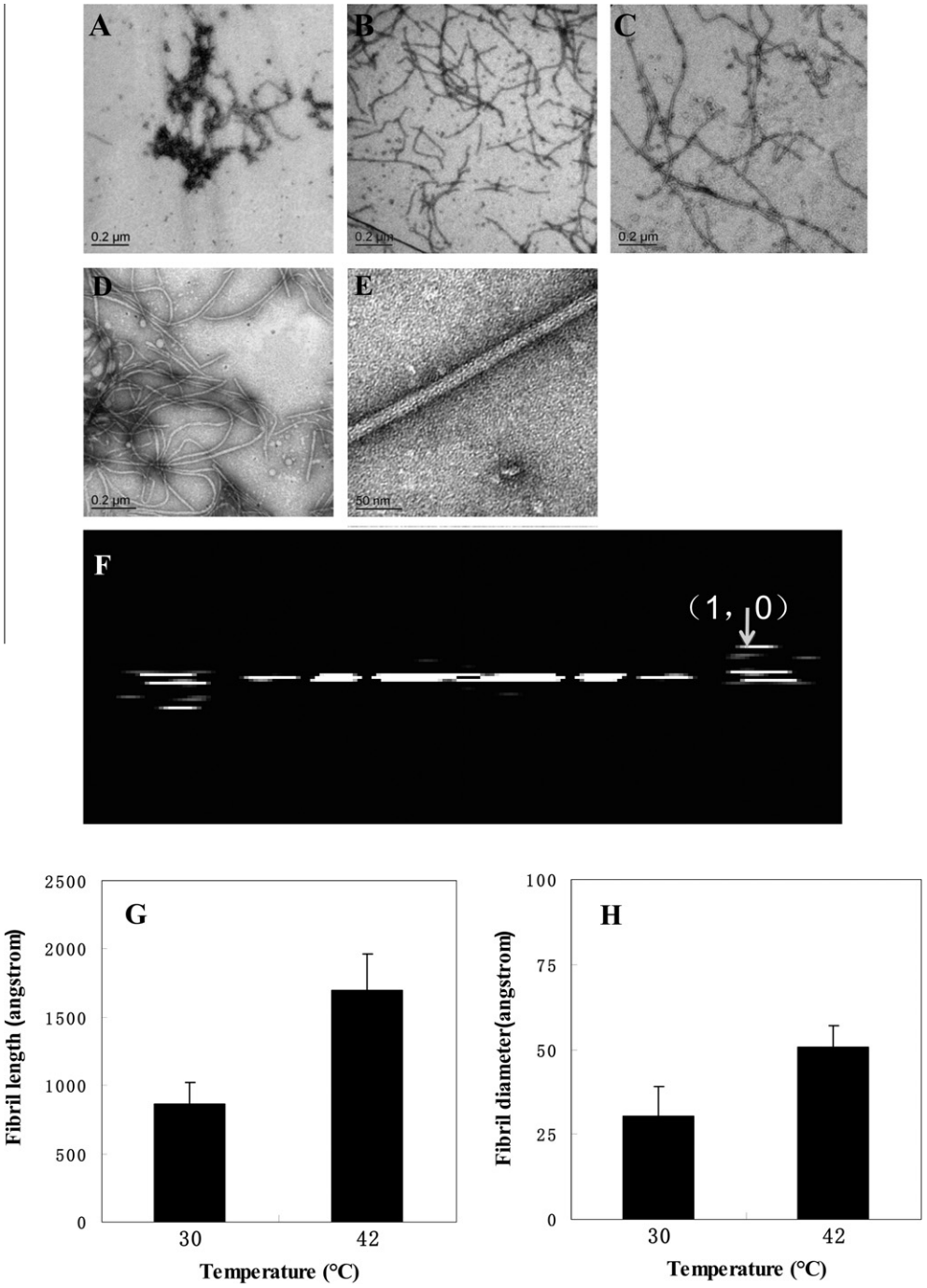

Fig. 1. AgsA fibril formation *in vitro* at different temperatures. (A–D), representative electron micrographs of purified AgsA pre-incubated at 20, 37, 50 and 65 °C. (E), details of an AgsA fibril at 20 °C. Pictures were taken on JEOL 1230 at a magnification of 20000×g. (F), Fourier transform of AgsA fibrils formed at 20 °C with the layer line (1, 0) index indicated. G and H describe the length and the diameter of AgsA fibrils, respectively, at 30 °C and 42 °C.

3. Results

3.1. AgsA forms fibrils at different temperatures

According to an earlier report by Tomoyasu et al. [15], large oligomers (about 440 kDa) of AgsA fail to dissociate upon a temperature increase from 25 to 50 °C, as revealed by SEC. Rather, a decrease of such large oligomers was observed. We confirmed these observations (Supplementary Fig. 1). In addition, no precipitates were observed for AgsA in buffer solutions after 30 min of heat treatment at 50 °C (data not shown). Therefore, we suggest that AgsA was partially transformed into other soluble structural forms that could not be eluted from SEC column. To test this idea, the AgsA proteins pre-incubated at different temperatures were examined by electron microscopy. Surprisingly, apart from spherical oligomers, AgsA was found to exist as fibrils after incubation at temperatures ranging from 20 to 65 °C (Fig. 1, A–D). A higher magnification micrograph taken at 20 °C (Fig. 1E) shows the details of AgsA fibril. Fourier transform of these fibrils gives diffraction pattern of layer lines, indicating that the AgsA fibrils have helical symmetry (Fig. 1F) [19].

Significant morphological changes were observed with AgsA fibrils as temperature increased. It was found that both the length and the diameter of AgsA fibrils exhibited a remarkable increase as the temperature increase from 20 to 65 °C (Fig. 1, A–D). Such an increase was also observed by quantitative measurements of twelve randomly selected fibrils at both 30 and 42 °C using cryo-electron tomography (Fig. 1, G and H). The actual data of these selected fibrils, i.e. their length and diameter, are presented in Supplementary Table 1. In addition, we found that fibril became the main structural form at high temperature (from 50 to 65 °C) (Fig. 1, C and D).

3.2. The fibrils formed by AgsA do not possess characteristics of amyloids

The fibril formed by AgsA is reminiscent of amyloids, a type of fibril associated with such neurodegenerative diseases as Alzheimer's and Parkinson's [20]. Amyloid formation is regarded as a general feature of polypeptide chains under harsh conditions [20]. Despite variations in morphology, amyloids mostly contain intermolecular β-sheets stacked perpendicular to the fibril axis, which is commonly characterized by such methods as ThT fluorescence assay and CR assay [20,21].

Data in this study demonstrate that the fibrils formed by AgsA are probably not amyloids. Results in Fig. 2A obtained from ThT fluorescence assay show a lack of increase in the fluorescence emission intensity at 490 nm when ThT was incubated with the AgsA fibrils formed at different temperatures. In contrast, a significant increase of fluorescence intensity was observed with amyloids of insulin, a molecule known to readily form amyloids, which bind to ThT, *in vitro* under harsh conditions [17]. Also, no binding of CR to the AgsA fibrils was observed (see Fig. 2B). Amyloids grown by lysozyme, a protein known to form amyloid under acidic conditions *in vitro* and whose amyloid binds to CR, were taken as the positive control [18]. In addition, Far-UV CD analysis showed that AgsA fibrils probably possess a somewhat lesser content of β-sheet than AgsA oligomers (see Fig. 2C). The results of Far-UV CD analysis showed that the spectra of AgsA solutions were characteristic of β-sheet structure, as indicated by a peak at 194 nm and a trough at 216 nm, which was consistent with the proposed structure of sHsps that is rich in β-sheet [22]. As the temperature rose from 20 to 65 °C, the CD spectra of the AgsA samples displayed a slight decrease in both the peak at 194 nm and the trough at 216 nm, indicating a mild decrease in the β-sheet content, with an observed increase in the abundance of AgsA fibrils upon such a temperature rise.

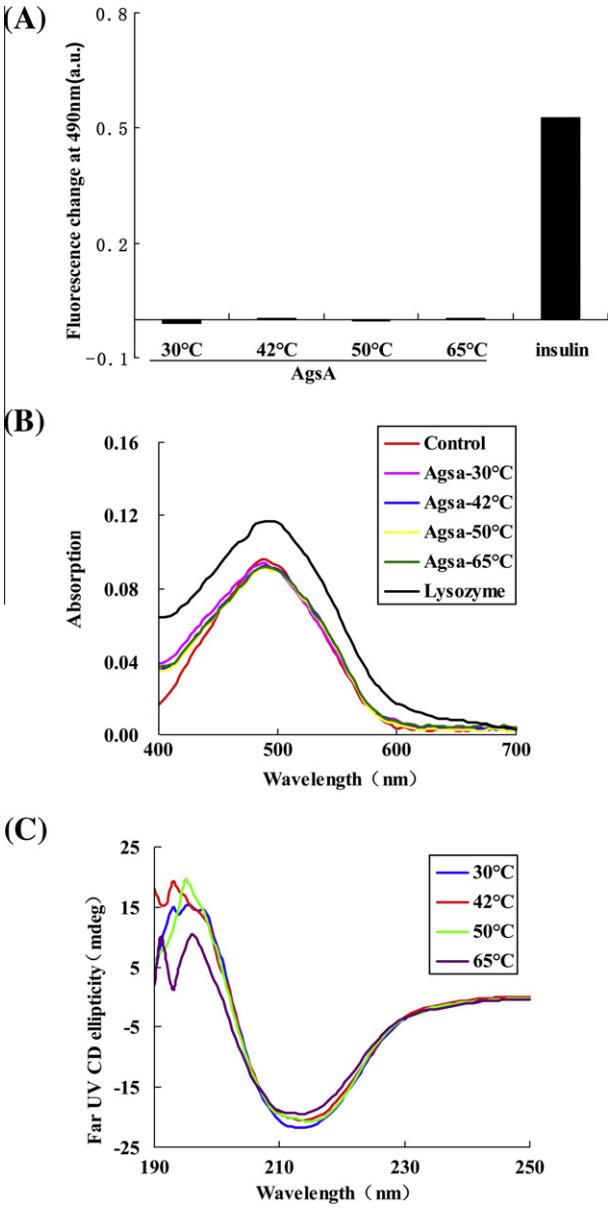

Fig. 2. (A) ThT fluorescence analysis of AgsA fibrils. The fluorescence emission intensity at 490 nm, excited by UV light at 442 nm, was recorded for fresh solutions of ThT with AgsA pre-incubated for 30 min at 30, 42, 50, 65 °C or insulin pre-incubated for 20 h at 50 °C. Fresh ThT solutions without proteins were taken as the negative control. Shown here are the changes in the fluorescence emission intensity at 490 nm from the negative control to the test groups. (B) CR assay of AgsA fibrils. The spectrum of CR alone (5 μM) was compared with that of CR solutions in the presence of protein (100 μg/ml), AgsA in solutions pre-incubated for 30 min at 30, 42, 50, 65 °C or lysozyme pre-incubated for a week at 37 °C, in 5 mM potassium phosphate buffer, 150 mM NaCl, adjusted to pH 7.4. Difference spectra are shown, which have been corrected for the contribution from buffer. (C) Far-UV CD analysis of AgsA samples at indicated temperatures.

3.3. Chaperone-like activity of AgsA is temperature-dependent

It is of interest to determine whether the fibril-forming AgsA would exhibit chaperone-like activity. Results in Fig. 3 demonstrate a sharp enhancement in AgsA chaperone-like activity upon a temperature increase from 25 to 55 °C. The data also suggest that AgsA showed almost no chaperone-like activity at temperatures below 25 °C and remained maximally active at temperatures between 42 and 55 °C.

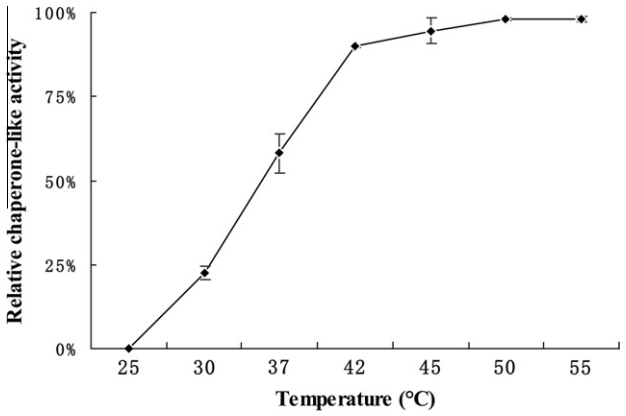

Fig. 3. Chaperone-like activity of AgsA is temperature-dependent. The relative chaperone-like activities, taken as the percentage of DTT-induced insulin aggregation inhibited by the presence of AgsA (final concentration of 0.5 mg/ml) at the indicated temperature, were calculated as ($A360_{insulin\ alone} - A360_{insulin+AgsA}$)/$A360_{insulin\ alone}$. Each experiment was repeated three times and one standard deviation is shown.

3.4. AgsA fibrils disappear in the presence of DTT-treated insulin

In order to further investigate a possible relationship between AgsA fibrils and chaperone-like activity, both of which displayed changes upon temperature shift, we investigated the morphological changes of AgsA fibrils upon the AgsA-mediated suppression of DTT-induced aggregation of insulin. Electron microscopy analysis showed that AgsA fibrils totally disappeared after incubated with DTT-treated insulin at 37 or 42 °C (Fig. 4A) or higher temperatures (data not shown). Meanwhile, a number of spherical particles were observed (Fig. 4A) and were found to be soluble. To characterize these particles, the same samples, i.e. the mixture of AgsA and DTT-treated insulin incubated at 42 °C, were analyzed by SEC followed by Coomassie brilliant blue staining. Results in Fig. 4B indicate that these particles are complexes formed between AgsA and insulin. The elution profile of the complexes formed between AgsA and insulin at 37 °C was the same with those formed at 42 °C (data not shown). In the absence of AgsA, DTT-treated insulin formed large aggregates at both 37 and 42 °C (Fig. 4A). Since AgsA proteins stayed in a soluble state at all the testing temperatures (data not shown), the possibility that AgsA fibrils might enter the precipitation should be eliminated.

4. Discussion

In general, sHsps, such as IbpB, Hsp16.5 and Hsp16.9, exist as multi-subunit spherical oligomers *in vitro* [23–25]. In this study, AgsA was revealed to exist as fibrils *in vitro* besides the conventional spherical oligomers. Notably, fibril formed by AgsA is a dynamic structure. First, it was observed that the length and the diameter of AgsA fibrils increased upon temperature elevation. Contrary to the property of a number of sHsps, whose oligomers disassemble into smaller units as temperature rises [11,12,26–28], AgsA not only maintains its large oligomers, but also assembles into fibrils with larger structural entities, i.e. longer and thicker fibrils. In addition to the morphological changes, there is an increase in the abundance of fibrils and a decrease in spherical particles upon temperature upshift. This observation indicates a possible structural switch at high temperature, in which AgsA spherical particles are converted to fibrils.

Fibril formation has been previously reported in two prokaryotic sHsps (IbpA of *E. coli* and StHsp19.7 in *Sulfolobus tokodaii* [29,30]), as well as in the eukaryotic sHsp α-crystallin [16]. However, data in this study suggest that the AgsA fibrils may be fundamentally distinguished from those of IbpA and other sHsps for displaying potential chaperone-like activity, which indicates that the AgsA fibrils are probably biologically functional structures. AgsA exhibits strong chaperone-like activity at 50 °C and above, where the majority of AgsA proteins exist as fibrils. An investigation into such a process by EM revealing the disappearance of AgsA fibrils in the presence of DTT-treated insulin further suggests the participation of AgsA fibrils in inhibiting insulin aggregation. On the contrary, though proved to interact with partially folded client proteins, the IbpA fibril has been found to only

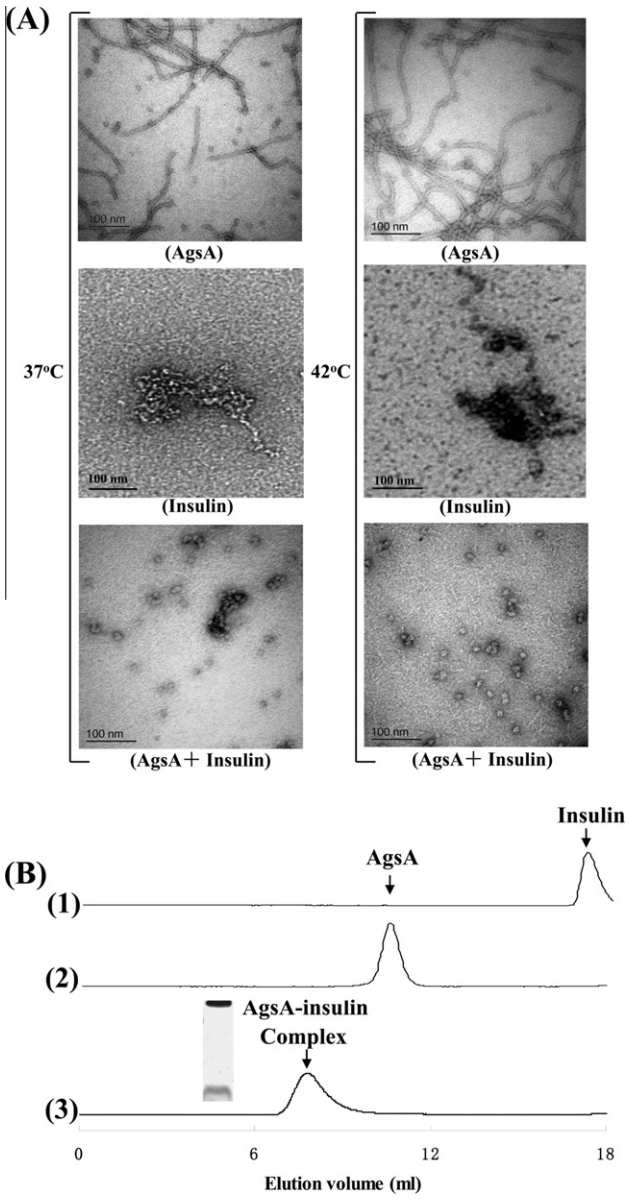

Fig. 4. (A) AgsA fibrils disappear in the presence of DTT-treated insulin. Samples of AgsA, DTT-treated insulin and mixture of pre-incubated AgsA and DTT-treated insulin were incubated at 37 or 42 °C for 30 min. Subsequently, samples were centrifuged at 15000×*g* for 1 min at 4 °C, and then stained and viewed. Pictures were taken on JEOL 1230 at a magnification of 20000×*g*. (B) Characterization of the particles of AgsA–insulin complex. Curves (1) and (2) show the size-exclusion chromatography analysis of insulin and AgsA alone respectively at room temperature. Curve (3) shows the elution profile of pre-incubated AgsA (0.5 mg/ml) mixed with insulin of the same concentration in the presence DTT (20 mM) at 42 °C. The positions of insulin, AgsA and AgsA–insulin complex are indicated by arrows. Inserted is the result of SDS–PAGE analysis of AgsA–insulin complex.

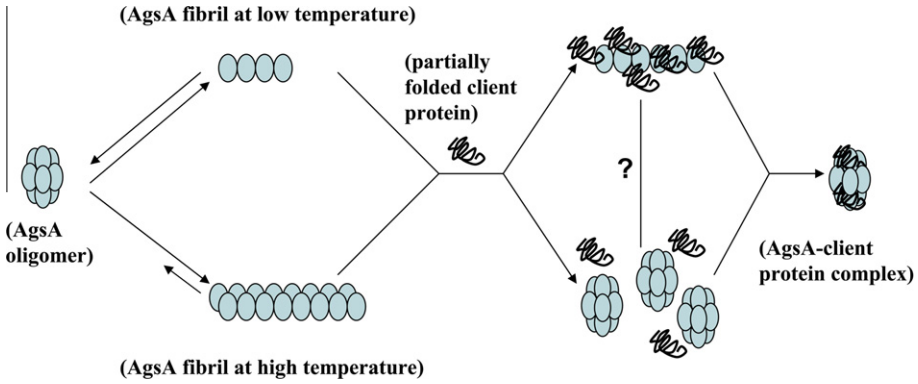

Fig. 5. Model illustrating the dynamic properties of AgsA fibrils at 50 °C and lower temperatures. At low temperature, AgsA forms shorter and thinner fibrils, which are in equilibrium with AgsA oligomers. In comparison, though also co-existing with AgsA fibrils at high temperature, AgsA oligomers have a greater tendency of switching into fibrils, which are longer and thicker. AgsA fibrils disappear in the presence of partially folded client proteins, which conversely results in the formation of spherical AgsA–client complexes. Two possible mechanisms are presented concerning the interactions between AgsA fibrils and client proteins. On the one hand, AgsA fibrils may be converted to oligomers before binding to the client proteins. On the other hand, AgsA fibrils may directly interact with the client proteins and subsequently give rise to AgsA–client protein complexes.

change the morphology of the aggregates of partially folded client proteins without preventing the formation of these aggregates. Therefore, IbpA fibril is proposed to be an inactive and inert form of this chaperone [29]. Similarly, fibrils formed by StHsp19.7 have also been revealed to lack chaperone-like activity [30]. Different from the amyloid-forming α-crystallin [16], fibrils formed by AgsA do not share characteristics of amyloids, as indicated by their inability to bind ThT and CR, which is similar with the property of IbpA fibrils [29]. Also, there is a lack of increase in the content of β-sheet, which is characteristic of amyloids, as the abundance of AgsA fibril increases [20]. Although the length and the diameter of AgsA fibrils change with temperature, the appearance of these fibrils remains essentially the same. These AgsA fibrils have an appearance similar to microtubules, which are different from that of amyloid fibrils. Amyloid fibrils are often twisted and result in regular "crossovers" that are visible by transmission electron microscopy [31]. All analyzed superhelical Aβ(1–40) and Aβ (1–42) fibrils have a left-handed twist and usually possess a polar structure [32–35]. However, AgsA fibrils are mostly single-stranded without twists or "crossovers".

Based on the above findings, a model is proposed to illustrate the dynamic properties of AgsA fibrils at 50 °C and lower temperatures (Fig. 5). In the absence of partially folded client proteins, the exposure of hydrophobic surface on AgsA molecules gives rise to the mutual affinity among them mediated by the hydrophobic force, thus facilitating their alignment into fibrils. Heat treatment probably increases hydrophobic exposure on the surface of AgsA, which results in the assembly into longer and thicker fibrils. The formation of fibril structures may prevent AgsA proteins from heat-induced irreversible aggregation, while retaining the potential chaperone function. In the presence of partially folded client proteins, AgsA fibrils disappear and AgsA–client complexes are formed. Detailed mechanisms of AgsA–client interaction remain to be elucidated. Here, two possible mechanisms are proposed based on the findings in this study. AgsA fibrils are in equilibrium with AgsA oligomers, which are ready for interacting and forming complexes with the partially folded client proteins. If so, AgsA fibrils, which are able to switch into oligomers to exhibit chaperone-like activity, may serve as a reservoir of functional AgsA proteins. Alternatively, the AsgA fibrils themselves may be chaperone active and able to interact with partially folded client proteins, resulting in complexes which are non-fibrillar in form.

In summary, this study shows that AgsA forms dynamic fibrils in vitro. Notably, these fibrils do not share characteristics of amyloids and possess potential chaperone-like activity within a certain range of temperature.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.09.042.

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